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Enhanced activity of horseradish peroxidase in Langmuir–Blodgett films of phospholipids

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ABSTRACT

The immobilization of enzymes in nanostructured films has potential applications, e.g. in biosensing, for which the activity may not only be preserved, but also enhanced if optimized conditions are identified. Optimization is not straightforward because several requirements must be fulfilled, including a suitable matrix and film-forming technique. In this study, we show that horseradish peroxidase (HRP) has its activity enhanced when immobilized in Langmuir–Blodgett (LB) films, in conjunction with dipalmitoylphosphatidylglycerol (DPPG). Incorporation of HRP into a DPPG monolayer at the air–water interface was demonstrated with compression isotherms, and Polarization-Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS). From the PM-IRRAS data, we inferred that HRP was not denatured when adsorbed on a pre-formed, low pressure DPPG monolayer. A change in orientation was induced by the phospholipid matrix, with the amide C=O and NH groups from HRP being oriented perpendicular to the surface, parallel to the DPPG acyl chains, i.e. the α-helix was inserted into the monolayer. The mixed DPPG-HRP monolayer could be transferred onto solid supports, to which HRP activity was ca. 23% higher than in solution. The control of molecular architecture and choice of a suitable phospholipid matrix allowed HRP-containing LB films to be used in sensing peroxide.

1. Introduction

Langmuir monolayers and Langmuir–Blodgett (LB) films have been extensively used over the years in connection with biomolecules and/or biologically-relevant molecules[1–4]. Perhaps the initial motivation was the use of phospholipid monolayers as models for a cell membrane[4–9], which in spite of the severe simplifications, provides molecular-level information that cannot otherwise be obtained. This is the case of the interaction between guest molecules, including peptides, proteins, pharmaceutical drugs, and phospholipids at distinct degrees of packing[10–16]. Relatively sophisticated information can be learnt from the experiments with Langmuir monolayers, e.g. the precise location of a drug in relation to the phospholipid molecules and the cooperative response from the monolayer molecules in the presence of minute quantities of drugs[13–15] or peptides [10,16]. Studies of these interactions have evolved from purely experimental work to molecular dynamics simulations, which are able to explain the effects from pharmaceutical drugs [15] and peptides [16] on the model membranes.

In addition to this interest in mimicking the cell membrane, Langmuir monolayers and LB films have also been used to investigate molecular recognition processes [2,17] and for biosensing [1,18]. Indeed, there exist various experimental techniques suitable to identify molecular recognition between film components and guest molecules, either co-spread at the air/water interface [19] or adsorbed from the aqueous subphase [20]. Leblanc [2], for instance, has discussed the "lock and key" interactions and the use of Langmuir monolayers as a model to understand molecular recognition-based biological assemblies. Other examples of molecular recognition in Langmuir monolayers include dynamic chiral recognition of amino acids by a polycholesteryl-substituted cyclen complex [21]. With regard to biosensing, in general proteins, enzymes in particular, have been immobilized in LB films that serve as sensing units for detecting various analytes. Because of the variety of properties exhibited by the immobilized biomolecules, biosensing may be performed with electrical measurements [22], electrochemical methods [23] and optical measurements [20].

A common feature in the three main uses of monolayers and LB films mentioned above is the need to preserve activity of the biomolecules. Here, advantage can be taken of the molecular control provided by the LB method, in which a suitable orientation of the biomolecule, induced by interactions in the film, may even yield bioactivities that are higher than for the same biomolecule in a homogeneous medium [24]. Since proteins adsorbing at an air/water interface on their own are likely to be denatured, a convenient matrix may be required. For instance, according to Girart–Egrot et al. [1], enzyme bioactivity in mixed lipid LB films is preserved because the

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lipid molecular assembly protects the enzyme, positioning the polypeptide moiety in such a way as to allow the recognition and signal events. In fact, phospholipids have been used as protecting agents for several types of material, not only for membrane cell proteins [25], but also for polysaccharides [26,27] and synthetic polymers [28].

In this study, we investigate activity preservation and biosensing with Langmuir monolayers and LB films of horseradish peroxidase (HRP). Peroxidases are oxide-reductase enzymes with hydrogen peroxide as electron acceptor [29], whose catalytic properties may be controlled by the microenvironment because the catalytic site contains a heme group that is highly sensitive to its vicinity[30–33]. HRP is extracted from a hard perennial herb, whose roots are important for culinary [29]. In a recent paper [34], we addressed the strategies of incorporation of HRP in lipid films at the air–water interface. Several methods of co-mixing were used, which resulted in different surface behaviors and micro-heterogeneities. Maximum incorporation of HRP occurred with the "injection method", i.e. the enzyme solution was injected in the subphase under a pre-formed lipid monolayer, which is the closest to real situations where drugs are injected in biological environments to interact with membranes. An important issue still to be addressed is whether HRP has its conformation preserved at the air–water interface in contact with lipid molecules, and whether HRP can be transferred onto solid supports in the form of LB films. Here we employ surface pressure, surface potential isotherms and Polarization-Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS) to confirm HRP adsorption on a phospholipid film. Significantly, we show that HRP incorporated in LB films transferred with these mixed monolayers exhibits a higher activity than in solution, thus being promising for sensing hydrogen peroxide.

2. Materials and methods

Dipalmitoylphosphatidylglycerol (DPPG) and horseradish peroxidase (HRP) were purchased from Sigma. The other chemicals were of the highest purity available. DPPG solutions (0.5–1.0 g/L) in chloroform:methanol (4:1 in volume) were spread on a 0.05 g/L phosphate buffer with ionic strength controlled using 0.01 mol/L NaCl and pH 6.3 in a mini-KSV trough with subphase volume of 220 mL. DPPG was chosen because of the abundance of lipids with glycerol polar heads in natural cell membranes and because it has opposite charge to HRP, which is positively charged under the conditions used in this paper. For producing mixed DPPG-HRP Langmuir monolayers, a total mass of 40 μg of HRP and 75 μg of DPPG was used, corresponding to a lipid: enzyme ratio of 99:1 (mol:mol) in the trough. The surface pressure was measured with the Wilhelmy method, with monolayer compression performed using two barriers at a 10 cm^2/min rate to obtain surface pressure–area isotherms. Surface potential was measured with a Kelvin probe. All measurements were carried out at 23±1 °C with water purified by a Milli-Q® system (18.2 MΩ cm and pH 6.3). The method for HRP incorporation is referred to as *injection method* [34], whereby a HRP solution was injected in the subphase after DPPG had been spread.

Polarization-Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS) was performed using a KSV PMI 550 instrument (KSV instrument Ltd, Helsinki, Finland). The Langmuir trough is set up so that the light beam reaches the monolayer at a fixed incidence angle of 80°. At this angle, the intensity is at maximum and the noise level is the lowest. A negative band indicates a transition moment oriented preferentially perpendicular to the surface, whereas a positive reflection absorption band indicates a transition moment oriented preferentially in the plane of the surface. The incoming light is continuously modulated between s- and p-polarization at a high frequency, which allows simultaneous measurement of the spectra for the two polarizations. The difference thus provides surface specific information, and the sum provides the reference spectrum. As the spectra are measured simultaneously, the effect of water vapor is largely reduced.

The transfer of the monolayers onto different solid supports (see below) was done at a surface pressure of 30 mN m^{-1} by emerging the solid substrate perpendicularly to the interface at a dip rate of 5 mm min^{-1}. The formation of LB films was confirmed firstly by analyzing the transfer ratio, and then using fluorescence spectroscopy (Shimadzu FR-5301PC), UV-vis spectroscopy (Hitachi U 2001), PM-IRRAS (KSV instrument Ltd, Helsinki, Finland) and a quartz crystal microbalance (QCM, Stanford Research Systems Inc.). The solid supports used were optical glass for fluorescence and UV-vis measurements, gold for PM-IRRAS, while for QCM we employed an AT-cut quartz crystal coated with Au, Stanford Research Systems Inc, fundamental frequency of ca. 5 MHz. For determining the enzyme activity, we inserted the solid support in a media containing pyrogallol at 12.8 mmol/L and hydrogen peroxide at 5 mmol/L, and the absorbance was monitored with time at 420 nm, according to the method described elsewhere [35]. All the experiments were repeated at least three times.

3. Results and discussion

3.1. Monolayers at the air–water interface

Fig. 1 shows PM-IRRAS data pointing to the incorporation of HRP injected in a buffer solution onto a lipid-free air–water interface. The main bands appear at 1554 and 1663 cm^{-1}, being assigned respectively to C=O stretching (amide I) and NH bending (amide II), which are typical of polypeptides. The infrared signal increases with time owing to increasing incorporation of HRP, which can also be observed in the adsorption kinetics (inset of Fig. 1), where the surface pressure increased from 0 to 10 mN/m within 60 min. The surface pressure stabilized at 11.5–12 mN/m within 90 min, after which there was negligible change in surface pressure and in the infrared bands. A small

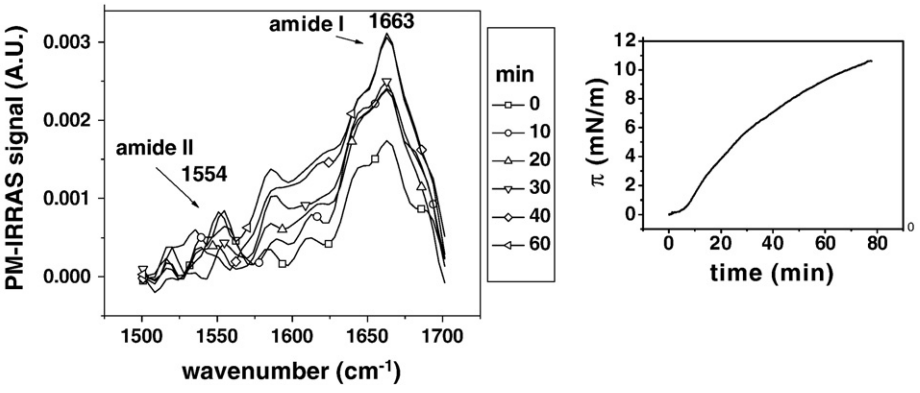

Fig. 1. PM-IRRAS spectra for HRP adsorbing at the air–water interface from a buffer subphase. The adsorption kinetics is shown in the right.

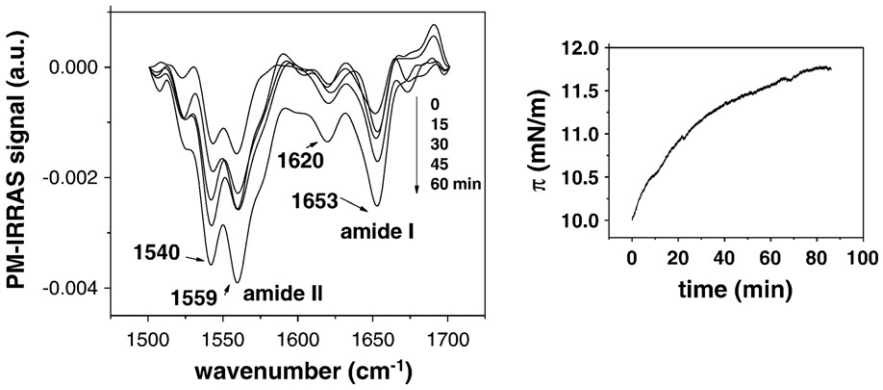

Fig. 2. PM-IRRAS spectra for HRP adsorbing at a DPPG monolayer (initial surface pressure of ca. 10 mN/m) from a buffer subphase. The adsorption kinetics is shown in the right.

band assigned to C=O stretching of β–sheets appears at 1619 cm^{-1}, much less intense than the band at 1663 cm^{-1}. The position of the amide I band in α-helical structures should lie between 1656 and 1649 cm^{-1} for native proteins [36]. As the value found here was 1663 cm^{-1}, it is probable that HRP may have been denatured when adsorbed at the air–water interface. This is common for proteins adsorbing at air–water interfaces because the polypeptide chain has to expose hydrophobic moieties towards the air [37]. Also, the strong positive amide I in comparison to amide II band indicates that the helix axis is parallel to the interface [38]. The bands for heme-C=O stretch in the 1900–1950 cm^{-1} region [39] are very weak, within the dispersion of the data, their analysis being therefore disregarded in this paper.

When HRP adsorbed at a pre-formed DPPG monolayer, the amide bands in Fig. 2 also changed with time due to HRP incorporation, as shown in the adsorption kinetics depicted in the inset. However, the bands characteristic of HRP are now inverted in addition to being better resolved in comparison to those for HRP adsorbing at a clean air/water interface. The inversion in the signal of amide bands has already been reported for β-lactoglobulin[40,41]. When the angle of incidence is above the Brewster angle, as in the present case (80°), positive intensities suggest a transition moment preferentially oriented in the plane of the surface, while negative bands indicate a perpendicular orientation [38]. Therefore, the amide C=O and NH groups from HRP are oriented preferentially perpendicular to the membrane surface, parallel to the relatively well-ordered acyl chains of DPPG, i.e. the polypeptide α-helix axis is inserted into the lipid monolayer. In other words, the presence of lipids at the air–water interface induced orientation changes in the HRP adsorbed. Further evidence of changes in orientation of HRP was provided by the splitting of the amide II into two bands (1540 and 1559 cm^{-1}), where the band at 1540 cm^{-1} indicates N–H deformation in β-sheet [42]. Better defined β-sheets were also inferred from the amide I bands centered at 1620 and 1653 cm^{-1}. All these data point to a change in orientation of HRP with phospholipids, with β-sheet structures arising due to the interaction of hydrophobic moieties of HRP with acyl chains of DPPG. Furthermore, HRP has probably not been denatured, as indicated by the main amide I band shifted to 1653 cm^{-1} in the presence of DPPG, which fits in the range reported for non-denatured proteins [43].

The incorporation of HRP was observed for other initial surface pressures of the DPPG monolayer, with the same behavior described above. However, no HRP was incorporated for surface pressures equal to or higher than 30 mN/m, which was denoted by absence of changes in surface pressure and in the amide bands. The pressure of 30 mN/m corresponds, therefore, to the exclusion surface pressure [34], above which it is no longer possible for HRP to be incorporated from a subphase into a compact monolayer.

In order to infer if the enzyme molecules remain at the interface when subjected to monolayer compression, after being adsorbed on a DPPG monolayer, we performed PM-IRRAS for DPPG alone and with the enzyme incorporated at 0 mN/m. Fig. 3 shows the data for DPPG without the enzyme. The surface pressure–area isotherm is typical for DPPG under the conditions used in this work, with a minimum area of 45 $Å^2$, and a liquid-condensed state between 50 and 40 $Å^2$. The surface potential–area isotherm shows a critical area of 65 $Å^2$ and a maximum potential of about 300 mV. A negative potential of ca. −100 mV at large areas appears due to the electric double-layer contribution [44], which occurs because the DPPG polar heads are ionized. Panel B in Fig. 3 shows the PM-IRRAS spectra for the C–H stretching mode region, dominated by two strong bands at 2917 and 2849 cm^{-1}, ascribed to the antisymmetric and symmetric CH_2 stretching vibrations, respectively. The antisymmetric band is slightly shifted to lower wavenumbers with increasing pressure. According to Dicko et al. [45], this is due to an increased number of *trans* conformers along the lipid chains, which correlates with increasing order upon monolayer compression.

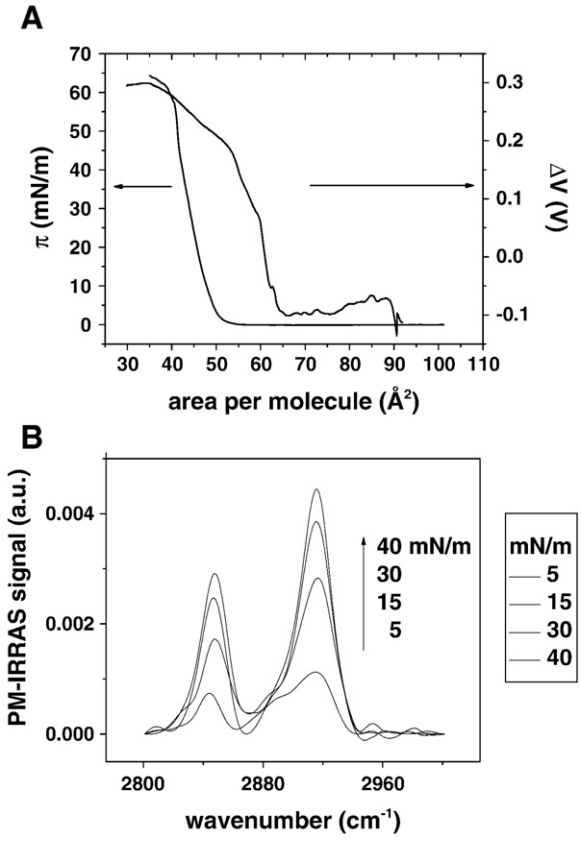

Fig. 3. Compression of DPPG monolayer on buffer–air interface. (A) π-A and ΔV-A isotherms. (B) PM-IRRAS spectra for CH vibration region.

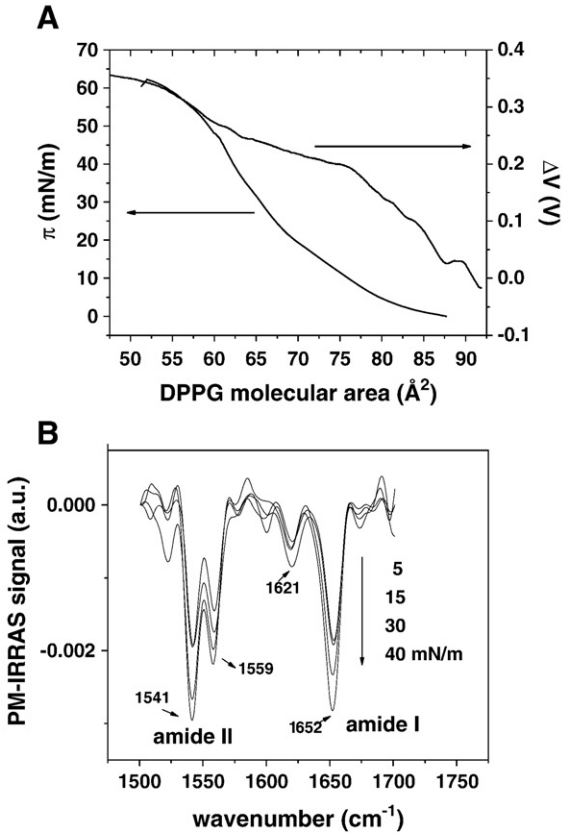

Fig. 4. Compression of a mixed HRP-DPPG monolayer on buffer–air interface. (A) π-A and ΔV-A isotherms. (B) PM-IRRAS spectra for amide vibration region.

Fig. 4 shows that HRP causes the monolayer to expand, with the liquid-expanded to liquid-condensed phase transition occurring at larger areas, consistent with a previous report [34]. The compressibility of the mixed monolayer is also higher than for pure DPPG, making it easier to compress the monolayer, as discussed previously [34]. The surface potential at large areas is positive in the presence of HRP, as the negative contribution from the double-layer is compensated for with the incorporation of the positively charged HRP. Upon compression the surface potential increases smoothly up to 320 mV. Because the HRP monolayer adsorbed without DPPG and when subjected to compression exhibited a surface potential close to 350 mV, the maximum surface potential is an average between the value of pure lipid and HRP.

It seems that HRP is not expelled from the interface even at high surface pressures during compression, for the monolayer remains expanded close to the collapse, which is corroborated by PM-IRRAS spectra. CH_2 bands are centered at 2849 and 2920 cm^{-1}. The higher wavenumber for antisymmetric CH_2 stretching in comparison to pure DPPG indicates the disorder induced by HRP adsorption. For the amide bands, the presence of HRP is noted even at high pressures (40 mN/m). Therefore, if adsorbed onto a DPPG monolayer at zero pressure, HRP is not expelled upon compression, even though it is not able to adsorb from the subphase on a compact (>30 mN/m) monolayer. The inverted peaks when compared to CH_2 indicates again that the amide C═O and NH groups are oriented preferentially perpendicular to the CH_2 stretching mode, and therefore, perpendicular to the interface. Only minor changes are observed when we compare the spectra from Figs. 2 and 4, indicating that HRP does not undergo considerable changes in its secondary structure under compression.

3.2. HRP incorporated in LB films

In the last section we mentioned that HRP could adsorb on a lipid-free air/water interface, thus forming a Gibbs monolayer. But the maximum surface pressure achieved was rather low, and the attempts to transfer such a film onto a solid support failed. HRP could be transferred though when mixed with DPPG. Mixed HRP-DPPG LB films were transferred onto solid supports at a surface pressure of ca. 30 mN/m, with the transfer of the enzyme being confirmed with nanogravimetric and spectroscopic methods. With a QCM, for instance, the mass of HRP-DPPG transferred in one layer was 700 ng, from which 210 ng should be attributed to HRP since the transfer of a neat DPPG layer yielded a mass of 490 ng. Considering the area of the solid support, this mass corresponds to an enzyme density of 230 ng/cm^2, leading to an area per HRP molecule of 2610 $Å^2$, where a molecular weight of 44,000 Da was used for HRP [31]. This value agrees with 2800 $Å^2$ determined for HRP on Si wafer by means of fluorescence measurements [46], or 2900 $Å^2$ [31], determined by X-ray, suggesting that HRP is adsorbed as a monomolecular layer together with one-layer DPPG LB film. To further predict the orientation of HRP molecules on the solid support, we use the dimensions of an ellipsoid HRP (40, 67 and 117 $Å^2$ [47]) and obtain projected areas varying from 2680 to 7840 $Å^2$. The lower value fits well to that found here (2610 $Å^2$) and suggests that HRP adsorbed on a DPPG monolayer has its major axis of the ellipsoid oriented perpendicularly to the phospholipids matrix, as depicted in Fig. 5.

The scheme in Fig. 5 is consistent with the PM-IRRAS results, from which the C=O and NH groups were inferred to be preferentially perpendicular to the water surface. Tang et al. [32] showed that HRP has its conformation changed in contact with DMPC vesicles, to adopt a conformation similar to that in Fig. 5, with the dimension of HRP molecule in contact with the liposome surface varying between 40 and 50 $Å^2$. Therefore, we may assume that HRP adapts to the lipid environment.

Fig. 6 shows the UV-vis spectra for HRP. For the solution, three main bands appear at 210, 275 and 400 nm, attributed respectively to π–π* and n–π* transitions of amide groups, and an electronic transition for

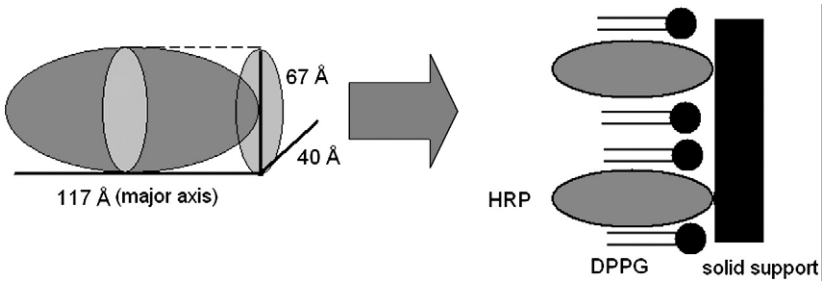

Fig. 5. Schematic model for HRP deposition on DPPG LB films. HRP is represented as an ellipsoid with major and minor axes as shown on the left. The mixed HRP/DPPG LB film is represented in such a way that the major axis is oriented perpendicularly to the solid support, according to quartz crystal microbalance results. The size of the components was not taken to scale to facilitate visualization.

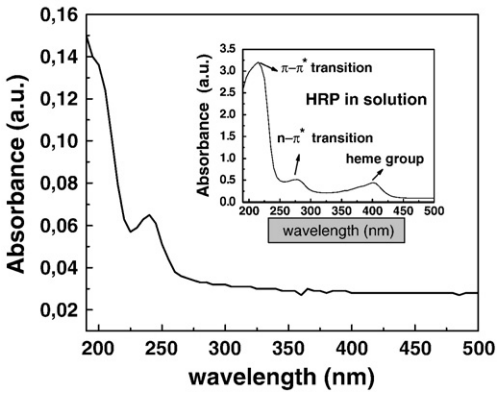

Fig. 6. UV-vis spectra for mixed HRP-DPPG LB film. The inset shows the UV-vis spectrum for HRP in solution (1 μg/mL).

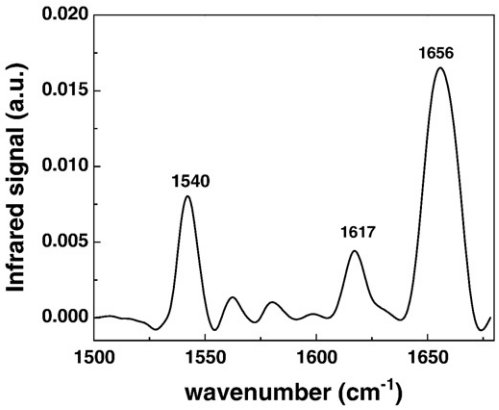

Fig. 8. Infrared spectra for mixed HRP-DPPG LB film.

the heme group in HRP. For the LB film, the π–π* and n–π* bands are shifted to lower wavelengths, 205 and 240 nm respectively. The band assigned to the heme group is not apparent in the spectrum for the LB film, probably because of the small mass transferred or due to the re-orientation of the enzyme upon immobilization. Indeed, Huang et al. [33] showed this band to be highly sensitive to hydrophobic environments. In the fluorescence spectra obtained with excitation at 280 nm in Fig. 7, emission due to the tryptophan groups occurs at 335 nm, and this band is shifted to ca. 375 nm for the LB film. A similar shift was observed for urease in the presence of phospholipids [20], which is attributed to a re-orientation of the polypeptide moiety and tryptophans induced by immobilization in a phospholipid matrix. The transfer of HRP was confirmed in PM-IRRAS measurements, with the amide II group appearing as a single main signal centered at 1540 cm^{-1}, while the amide I had an α-helix component at 1656 cm^{-1} and a β-sheet component at 1617 cm^{-1} (Fig. 8).

3.3. HRP activity

Immobilization of enzymes in solid matrices is a challenge in terms of preservation of catalytic activity. The solid support and way of immobilization should avoid enzyme denaturing and provide an orientation and surface density that maximize catalytic activity [12]. In this context, the microenvironment in which the enzyme is entrapped plays an important role in keeping the conformation and orientation of the polypeptide chain. The geometry of the immobilized enzyme molecule is important not only for the overall conformation in terms of secondary and tertiary structures, which is essential for the biological functions, but also for a suitable positioning of the catalytic site for access of the substrate (analyte).

Denaturing of enzymes immobilized in solid matrices is common, as the enzymes strive for achieving a lower surface energy, or may not adequately expose their catalytic sites [12,48]. On the other hand, reports have been made of higher activities for enzymes immobilized under mild conditions [49]. The activity of enzymes immobilized in lipid matrices, for instance, may be higher [20] or lower [12] than in a homogeneous environment. For enzyme entrapped in phospholipid molecules, as in an HRP-DPPG LB film studied here, the close packing of the enzyme adapted to a bio-inspired interface (phospholipids) can lead to a favorable conformation/orientation of the polypeptide structure, which enhances enzyme activity. Tight-packed films may induce a decrease in enzyme activity as reported for glucose oxidase [48]. However, when lipid films are used as adsorption matrix, material permeation through films can be enhanced.

The enzyme activity of HRP immobilized in DPPG LB films, shown in Fig. 9, was higher than in solution. Activity was detected using pyrogallol that reacts with hydrogen peroxide forming purporogallin, which absorbs at 420 nm. HRP thus catalyzes this oxydo-reduction reaction. By measuring the increase in absorbance within the first 20 minutes of reaction and using the mass of HRP adsorbed from QCM we obtained an enzyme activity of 1815 ng of purporogallin min^{-1} per mg of HRP. In this estimation, the extinction coefficient was 2470 mol^{-1} L cm^{-1} for the product [35]. In contrast, the activity of HRP in a homogeneous medium was 1480 ng of purporogallin min^{-1} per mg of HRP. Also significant was the stability of the enzyme activity measured. For even after 2 weeks with the HRP-DPPG LB film in a buffer solution at 10 °C, more than 90% of the initial enzyme activity was maintained.

HRP immobilized in poly(aniline), or in a poly(ethylene terephthalate)-poly(aniline) composite, retained 40–70% of the activity when

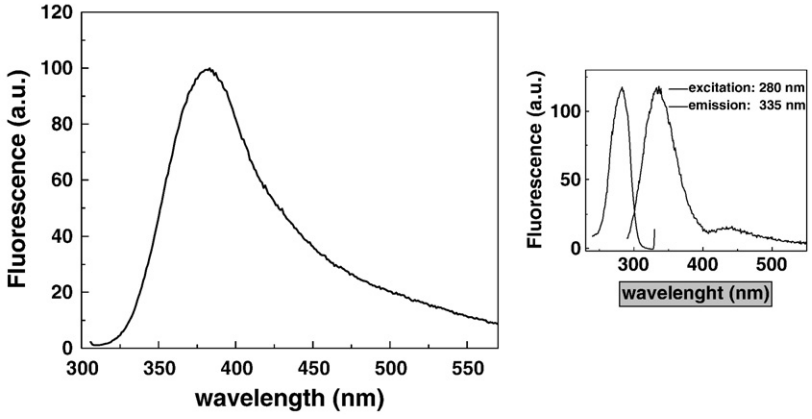

Fig. 7. Emission spectrum for a mixed HRP-DPPG LB film. The inset shows the emission–excitation fluorescence spectra for HRP in solution (1 μg/mL).

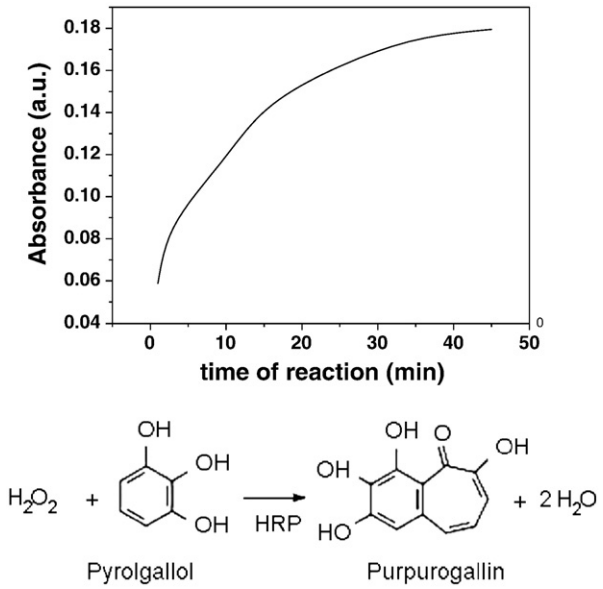

Fig. 9. Enzyme activity for mixed HRP-DPPG LB films using pyrogallol.

compared to HRP in solution [50]. Naves et al. [47] reported a loss of 50% of activity for HRP immobilized on silicon wafers, which was attributed to the difficult access of the analyte for immobilized HRP. Vianello et al. [46] immobilized HRP in several solid supports, and only 5% of the enzyme activity was found in comparison to the free enzyme. As far as LB films are concerned, HRP immobilized from solution in a pre-formed stearic acid LB film had only 30–40% of the free enzyme activity [51].

With regard to cases where HRP activity was reported to be higher than in solution, Gazaryan et al. [30] obtained micellar matrices for HRP that enhanced activity due to favorable hydrophobic and dipole interactions. This enhancement was ascribed to a screening of the catalytic site, with the micellar structures inducing superactive conformations and enzyme dimeric structures. Furthermore, interaction of glycosyl parts of HRP with hydrophilic heads of surfactants can induce the enzyme to reach its better conformation-orientation for adequate accessibility of the substrate for catalytic sites. Tang et al. [32] showed that DMPC vesicles with HRP incorporated makes the heme group of HRP in the lipid bilayer to achieve a favorable orientation for exchanging electrons, increasing activity. In other words, the lipid microenvironment facilitated electron transfer, and retained the HRP biological activity.

We may now elaborate on the reasons for an enhanced HRP activity in mixed LB films with DPPG. It seems that the DPPG LB film structure keeps the enzyme in a suitable orientation for access to the heme group involved in the redox reaction. In fact, Gajhede et al. [31] proposed that the substrate access to catalytic site is through an external channel, which has a peripheral hydrophobic layer comprising Phe residues and C18 methyl from the heme group. The inner channel contains an overall positively charged character due to distal Arg and Phe residues that form a distinctive hydrophobic patch near the exposed heme edge. Also, HRP has an extensive hydrogen-bonding network in the proximal and distal region of the heme. The distal pocket is connected to the proximal side by a hydrogen-bonding network. It is believed that hydrophobic interactions cause this channel to open by breaking the H-bonds, thus facilitating the access of the analyte to the active site. The conformation change of HRP might therefore occur because the DPPG membrane affects the hydrogen-bonding network around the heme in HRP. In comparison with the earlier work in which HRP activity was reduced when immobilized in an already-formed, tightly packed LB film [51], we observe that in our case HRP was adsorbed from the subphase at a fluid lipid Langmuir monolayer. This may have driven the adsorption and orientation of HRP molecules to penetrate between hydrophobic chains or simply interact with the polar head groups.

4. Conclusions

We demonstrated that HRP can adsorb onto Langmuir monolayers of DPPG, forming stable monolayers, and having the polypeptide orientation changed, according to PM-IRRAS data. HRP cannot be incorporated at the air–water interface at initial surface pressures above 30 mN/m, but can be retained when high surface pressures are reached upon compressing a mixed enzyme-DPPG monolayer formed at lower initial surface pressures. HRP could be transferred onto solid supports as LB films, as confirmed by QCM, UV-vis, fluorescence and infrared spectroscopy. Enzyme activity could be detected for mixed HRP-DPPG LB films, being higher than in solution. The good control of molecular architecture provided by the LB technique seems essential for using HRP films in peroxide sensors, whose viability was proven using colorimetric measurements.

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